A study on urinary biomarkers of tubular injury in Type-2 Diabetes Mellitus patients

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by

Sujatha R (MD Biochemistry)

(UIN 2005002004)

Under the guidance of

Dr Sumathi S

Professor and Head

Department of Biochemistry

Mahatma Gandhi Medical College and Research Institute, Puducherry

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INSTITUTIONAL HUMAN ETHICS COMMITTEE CLEARANCE

Institutional Human Ethics Committee
PSG Institute of Medical Sciences & Research
Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER, WHO)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : +91 422 - 4345818, Fax : +91 422 - 2594400, Email : ihec@psgimsr.ac.in
EC-CT-2018-0055

Ref. No.: PSG/IHEC/2022/Appr/Exp/201

February 14, 2025

To
Dr Sujatha R
Associate Professor
Department of Biochemistry
PSG IMS & R
Coimbatore
Guide: Dr Sumathi S
Co-investigators: Dr Vijaya D / Mrs Susipriya P / Ms Saju Denishya P / Ms Aathira S / Ms Dharshika S

Ref: Project No. **22/195**

Dear Dr Sujatha,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 14.07.2022 to conduct the research study entitled "*A study on urinary biomarkers of tubular injury in Type - 2 Diabetes Mellitus patients*" during the IHEC meeting held on 29.07.2022.

The following documents were reviewed and approved:

1. Project submission form
2. Study protocol (Version 1 dated 14.07.2022)
3. Application for waiver of consent
4. Confidentiality statement
5. Data collection tool (Version 1 dated 14.07.2022)
6. Permission letter from Director – Research and Innovation
7. Current CVs of Principal investigator, Co-investigators
8. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the expedited review meeting held on 29.07.2022 between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Dr Rajani Sundar (Chairperson, IHEC)	MD, DA	Clinician	Female	No	Yes
2	Dr S Karthikeyan (Member – Secretary, IHEC)	MD	Epidemiologist, Ethicist	Male	Yes	Yes
3	Dr S Ramesh	MD	Clinician, Paediatrics	Male	Yes	Yes
4	Mrs M Nirmala (Alt. member-Secretary, IHEC)	M Sc	Nursing	Female	Yes	Yes

The study is approved in its presented form for the stated sample size. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the New Drugs and Clinical Trials Rules, 2019. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

SECRETARY PSG IMS&R COIMBATORE-641004.

Proposal No. 22/195 dt.14.02.2025, Title: A study on urinary biomarkers of tubular injury in Type - 2 Diabetes Mellitus patients (Wrf. to PI;s letter dated 11.02.2025 for change of guide and inclusion of co-investigators, copy generated on 14.02.2025)

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STRUCTURED ABSTRACT

Introduction: Diabetic kidney disease (DKD) is a critical microvascular complication affecting 20-40% of diabetic patients globally. Given the rising prevalence of DKD, early identification is essential for timely intervention to prevent or delay the progression to end-stage renal disease. Traditional screening using urinary albumin-creatinine ratio (UACR) has significant limitations, as renal impairment can occur before microalbuminuria develops. Therefore, there is a critical need for more non-invasive urinary biomarkers that can enable early detection of DKD and accurately predict its progression to end-stage renal disease.

Objectives:

- Measure and compare levels of urinary biomarkers (Megalin, NGAL, Cystatin-c, and NAG) across different albuminuria stages
- Evaluate the diagnostic accuracy of individual and combined urinary biomarkers compared to UACR
- Investigate the influence of demographic and clinical factors on urinary biomarker levels
- Explore the relationship between urinary biomarkers and established renal function parameters

Methods: A cross-sectional study was conducted at PSG Institute of Medical Sciences and Research, Coimbatore, involving 320 T2DM patients aged 18 or older. Participants were categorized into three groups based on UACR: normoalbuminuria, microalbuminuria, and macroalbuminuria. Urinary biomarkers were measured using ELISA kits, and comprehensive biochemical and clinical parameters were analyzed. Statistical analysis included Kruskal-Wallis tests, ROC curve analysis, correlation studies, and logistic regression.

Results: NAG, Megalin, and NGAL showed significant differences across albuminuria groups. NGAL demonstrated the strongest diagnostic performance with an Area Under the Curve (AUC) of 0.764, sensitivity of 0.625, and specificity of 0.817. Multiple logistic regression revealed NGAL (OR = 1.892) and NAG (OR = 1.043) as strong independent predictors of albuminuria. Significant correlations were observed between NGAL and HbA1c, supporting the link between glycemic control and tubular injury.

Conclusion: Urinary tubular biomarkers, particularly NGAL and NAG, show promise in early detection of diabetic kidney disease. These markers can potentially identify renal injury before significant albuminuria develops, suggesting their utility in enhancing current diagnostic approaches. Longitudinal studies are recommended to validate these findings and establish clinical cut-off values.

Keywords: Diabetic Kidney Disease, Lipocalin, Low Density Lipoprotein Receptor-Related Protein-2, Albuminuria, Early Diagnosis

INTRODUCTION

Diabetic kidney disease (DKD) is a critical microvascular complication of diabetes mellitus (DM), affecting 20-40% of diabetic patients globally (1). As the leading cause of end-stage renal disease (ESRD), DKD significantly impacts patient health by increasing the risk of all-cause mortality, cardiovascular disease, and necessitating expensive renal replacement therapies such as dialysis or kidney transplantation.

Traditionally, the urinary albumin-creatinine ratio (UACR) has been the primary screening method for early DKD detection. However, emerging research reveals significant limitations in this approach. Renal impairment can occur before the development of microalbuminuria, and UACR measurements can be influenced by various factors including physical activity, urinary tract infections, acute illness, and heart failure, which reduce its diagnostic specificity (2).

The clinical landscape of type-2 diabetes mellitus (T2DM) presents a particularly challenging scenario. Studies indicate that 20-40% of T2DM patients may experience kidney dysfunction at the time of diagnosis, often before albuminuria becomes detectable (3). Normoalbuminuric T2DM patients with renal insufficiency are less likely to receive appropriate treatment adjustments compared to those with albuminuric kidney impairment, highlighting a critical gap in current diagnostic approaches.

Recent research has shifted focus towards alternative diagnostic strategies, particularly urinary biomarkers of tubular injury. These biomarkers offer promise in detecting kidney damage earlier than traditional methods. Preliminary studies suggest that urinary biomarkers can be elevated in normoalbuminuric T2DM patients, indicating their potential for early kidney injury detection (4).

This approach could potentially identify kidney damage at a much earlier stage, enabling more proactive and personalized medical interventions.

The growing interest in urinary biomarkers stems from their ability to provide insights into kidney health before significant structural changes occur. Markers such as Megalin, Neutrophil Gelatinase Associated Lipocalin (NGAL), Cystatin-c, and N-Acetyl Glucosaminidase (NAG) have shown potential in detecting subtle kidney damage that may be missed by conventional screening methods (5).

Considering this background of challenges and opportunities, our study aims to evaluate the potential of urinary tubular biomarkers in early detection of diabetic kidney disease. Comparison of these innovative biomarkers with the current gold standard of UACR, may assist in developing more sensitive and precise diagnostic approaches for identifying renal damage in T2DM patients.

AIM AND OBJECTIVES

Aim:

To evaluate the usefulness of urinary biomarkers of tubular injury (Megalin, Neutrophil Gelatinase Associated Lipocalin (NGAL), Cystatin-c, and N-Acetyl Glucosaminidase (NAG)) in early detection of diabetic kidney disease in type-2 diabetes mellitus patients.

Primary Objectives

- Measure and systematically compare levels of Megalin, Neutrophil Gelatinase Associated Lipocalin (NGAL), Cystatin-c, and N-Acetyl Glucosaminidase (NAG) across different albuminuria stages (normoalbuminuria, microalbuminuria, and macroalbuminuria) in T2DM patients
- Compare the diagnostic accuracy of individual and combined urinary tubular biomarkers with the current gold standard (UACR) by evaluation of sensitivity, specificity, Positive predictive value, Negative predictive value, Area under the ROC curve for each biomarker

Secondary Objectives

- Investigate the influence of demographic and clinical factors such as age, gender, duration of diabetes, HbA1c and lipid profile on urinary biomarker levels
- Explore the relationship between urinary tubular biomarkers and established renal function parameters such as serum urea, serum creatinine, estimated glomerular filtration rate (eGFR) by CKD-EPI 2021 and MDRD formulae.

HYPOTHESIS/RESEARCH QUESTIONS

Primary Research Question

"Are urinary tubular biomarkers (Megalin, NGAL, Cystatin-c, and NAG) more effective than UACR in early detection of diabetic kidney disease in Type-2 diabetes mellitus patients?"

Primary Hypothesis

H0 (Null): Urinary tubular biomarkers (Megalin, NGAL, Cystatin-c, and NAG) are not more effective than UACR in early detection of diabetic kidney disease in Type-2 diabetes mellitus.

H1 (Alternative): Urinary tubular biomarkers (Megalin, NGAL, Cystatin-c, and NAG) are more effective than UACR in early detection of diabetic kidney disease in Type-2 diabetes mellitus.

Secondary Hypotheses

1. Regarding Biomarker Levels Across Disease Stages

H0 (Null): There is no significant difference in urinary tubular biomarker levels across normoalbuminuric, microalbuminuric, and macroalbuminuric stages.

H1 (Alternative): There are significant differences in urinary tubular biomarker levels across normoalbuminuric, microalbuminuric, and macroalbuminuric stages.

II. Comprehensive Demographic and Clinical Correlation Hypothesis

H0 (Null Hypothesis): Demographic and clinical variables (age, gender, diabetes duration, HbA1c, and lipid profile) do not significantly influence urinary tubular biomarker levels in Type-2 diabetes mellitus patients.

H1 (Alternative Hypothesis): Demographic and clinical variables (age, gender, diabetes duration, HbA1c, and lipid profile) significantly influence urinary tubular biomarker levels in Type-2 diabetes mellitus patients.

III. Renal Function Parameters Hypothesis

H0 (Null Hypothesis): Urinary tubular biomarkers (Megalin, NGAL, Cystatin-c, and NAG) show no significant correlation with established renal function parameters in Type-2 diabetes mellitus patients.

H1 (Alternative Hypothesis): Urinary tubular biomarkers demonstrate significant correlations with established renal function parameters in Type-2 diabetes mellitus patients.

Expected Outcomes

1. At least one or more tubular biomarkers will show significant elevation in diabetic patients before the appearance of microalbuminuria.
2. Specific cut-off values for each biomarker will be established for early detection of diabetic nephropathy.
3. A composite score or algorithm combining multiple biomarkers will demonstrate superior diagnostic accuracy compared to individual markers or UACR.
4. Certain biochemical and clinical parameters (such as age, gender, HbA1c, disease duration, lipid profile, urea, creatinine, and eGFR) will show significant correlation with urinary tubular biomarker levels, potentially identifying high-risk subgroups.

REVIEW OF LITERATURE

Diabetic kidney disease (DKD) is a progressive microvascular complication of type 2 diabetes mellitus (T2DM), affecting both glomerular and tubular function. Chronic hyperglycemia induces oxidative stress, inflammation, and endothelial dysfunction, leading to renal structural and functional impairment (6). Traditionally, the pathophysiology of DKD has focused on glomerular injury, including mesangial expansion, podocyte loss, and basement membrane thickening, ultimately resulting in proteinuria and a decline in glomerular filtration rate (GFR). However, recent evidence suggests that tubular injury may precede glomerular damage, emphasizing the critical role of proximal tubular dysfunction in the early stages of DKD (7). Tubular cells are particularly vulnerable to hyperglycemia-induced injury due to their high metabolic activity and exposure to reabsorbed glucose and filtered proteins, which contribute to progressive tubulointerstitial fibrosis and nephron loss (6,7).

The tubular hypothesis of DKD underscores the role of proximal tubular epithelial cells in initiating and propagating renal injury. Hyperglycemia-driven mitochondrial dysfunction, activation of pro-inflammatory pathways, and increased oxidative stress disrupt normal tubular function, leading to impaired reabsorption and increased urinary excretion of tubular proteins. The accumulation of advanced glycation end-products (AGEs) and upregulation of transforming growth factor-beta (TGF-β) further promotes tubular fibrosis. While albuminuria has traditionally been used as an early marker of DKD, its limitations are evident, as renal function decline can occur even in normoalbuminuric individuals. This underscores the need for alternative biomarkers to detect early renal injury before overt proteinuria develops (8).

Several urinary biomarkers have been identified as indicators of tubular injury in DKD, providing insights into the underlying pathophysiological mechanisms. Megalin, a key endocytic receptor

expressed in proximal tubular cells, plays a crucial role in the reabsorption of filtered proteins. Tubular dysfunction results in impaired megalin-mediated endocytosis, leading to increased urinary excretion of megalin-bound proteins, serving as an early marker of tubular injury (9). Neutrophil gelatinase-associated lipocalin (NGAL), a siderophore-binding protein, is highly upregulated in response to tubular stress and ischemic injury, reflecting both acute and chronic renal damage (6,7). Cystatin C, a low-molecular-weight protein freely filtered by the glomerulus and reabsorbed by proximal tubules, is an established marker of GFR. Increased urinary cystatin C suggests proximal tubular dysfunction and impaired reabsorption (8). N-acetyl-β-D-glucosaminidase (NAG), a lysosomal enzyme released from damaged proximal tubular cells, serves as an indicator of tubular epithelial cell injury and inflammation (10).

The utility of these biomarkers extends beyond the detection of tubular injury, as they provide diagnostic and prognostic insights into DKD progression. Elevated urinary levels of these biomarkers have been observed even in normoalbuminuric diabetic patients, suggesting their potential role in identifying subclinical renal impairment. The ability to detect DKD in normoalbuminuric individuals is particularly relevant, as a significant proportion of diabetic patients exhibit progressive renal dysfunction despite the absence of albuminuria. Moreover, biomarker variability across different stages of DKD and among different demographic groups highlights the importance of establishing reference ranges and cut-off values tailored to specific populations (11).

Despite advancements in biomarker research, current clinical guidelines continue to rely on albuminuria and estimated GFR (eGFR) for DKD diagnosis and staging, potentially delaying early intervention. The integration of tubular biomarkers into routine clinical practice holds promise for improving early detection, risk stratification, and personalized therapeutic strategies. Given the

complex interplay between glomerular and tubular injury in DKD, a multimodal approach combining multiple biomarkers may enhance diagnostic accuracy and facilitate early nephroprotection. The identification of optimal biomarker cut-off values and their correlation with clinical parameters such as HbA1c, lipid profiles, and renal function markers could further refine their clinical applicability (12). Addressing these gaps in early DKD detection is critical to mitigating disease progression and improving long-term outcomes in diabetic patients.

In this regard, several studies have been conducted to evaluate urinary biomarkers of tubular injury in Type-2 Diabetes Mellitus.

Zhang C et al. (2024) investigated the distribution of nine urinary biomarkers in people with Type 2 Diabetes Mellitus (T2DM), with and without microvascular complications. A total of 407 T2DM patients were enrolled and categorized into four groups based on diabetic retinopathy (DR) and urinary albumin-creatinine ratio (UACR): DR(–)UACR(–), DR(+)UACR(–), DR(–)UACR(+), and DR(+)UACR(+). The nine biomarkers included u-α1MG, u-IgG, u-NGAL, u-CysC, u-RBP, u-β2MG, u-NAG, u-Trf, and u-Col. Results showed that u-NAG/Cr levels significantly differed between healthy participants and T2DM patients. In the DR(–)UACR(–) group, u-Trf/Cr had the highest positive rate (21.37%), followed by u-IgG/Cr (14.52%), and u-NAG/Cr (10.48%). Additionally, 17.34% of T2DM patients showed positivity for multiple biomarkers. The study concluded that renal tubular biomarkers, especially u-NAG, can be useful for early detection and monitoring of renal injury in T2DM patients, particularly at first-time diagnosis (13).

Korbut AI et al. (2023) evaluated urinary biomarkers of tubular injury and renal fibrosis in patients with type 2 diabetes (T2D) and chronic kidney disease (CKD) with non-albuminuric and albuminuric patterns. One hundred and forty T2D patients and 20 non-diabetic controls were included. Urinary levels of RBP-4, GST-α1, GST-π, TGF-β, Col1, Col4, BMP-7, and HGF were

assessed. T2D patients had higher excretion of RBP-4, GST-π, Col4, BMP-7, and HGF compared to controls. In patients with elevated albumin-to-creatinine ratio (UACR), RBP-4, GST-π, Col1, and Col4 were elevated. These markers correlated positively with UACR but not with glomerular filtration rate. Elevated excretions of RBP-4, GST-π, Col1, Col4, and HGF were associated with the albuminuric pattern of CKD in T2D (14).

Phanish MK et al. (2021) measured nine urinary biomarkers in 400 diabetic patients to assess their association with chronic kidney disease (CKD) and progression. In the ACR <3 cohort, N-acetyl-β-d-glucosaminidase was significantly linked to an estimated glomerular filtration rate (eGFR) < 60 ml/min. The combination of ACR and MCP1 was associated with stage 2 CKD, and biomarkers such as ACR, retinol binding protein (RBP), and MCP1 were linked to CKD progression across all levels of albuminuria. MCP1, RBP, and ACR were significant in linear and logistic models for progression to stage 3 or worse. The study concluded that ACR, RBP, and MCP1 were key biomarkers for CKD progression, with inflammatory cytokines and MCP1 also relevant in non-albuminuric patients (15).

Satirapoj B et al. (2019) conducted a prospective cohort study involving 257 patients with Type 2 Diabetes Mellitus (T2DM) and aimed to assess the association of urinary tubular biomarkers with rapid renal progression and the incidence of end-stage renal disease (ESRD). Biomarkers such as urine albumin, cystatin-C, angiotensinogen, kidney injury molecule-1 (KIM-1), and neutrophil-gelatinase associated lipocalin (NGAL) were measured at baseline. At a 3-year follow-up, 26.1% of patients experienced rapid glomerular filtration rate (GFR) decline or ESRD. The study concludes that elevated baseline levels of these urinary biomarkers are associated with a higher incidence of ESRD and faster GFR decline in T2DM patients (10).

METHODOLOGY

Study Design: This cross-sectional study was designed to investigate urinary biomarkers of tubular injury in Type-2 Diabetes Mellitus (T2DM) patients.

Study Location: PSG Institute of Medical Sciences and Research, Coimbatore, Tamilnadu, India

Study Period: Samples were collected over a period of two years (November 2022 to November 2024).

Ethical considerations: The study proposal was submitted to the Institutional Human Ethics Committee. The proposal was reviewed and since the left-over samples were used for the study purpose, waiver of consent was granted by the ethics committee. The study was approved in its presented form with approval number 22/195.

Study Population: The study included adult patients aged 18 years or older, diagnosed with T2DM according to the ADA criteria (16), and with a disease duration of at least 5 years.

Based on their urinary albumin creatinine ratio (UACR), participants were categorized into three groups: (17)

Normal albuminuria (UACR <30 mg/g creatinine)

Microalbuminuria (UACR 30-299 mg/g creatinine)

Macroalbuminuria (UACR ≥300 mg/g creatinine)

Sample Size Calculation: The sample size was calculated using nMaster 2.0 software, based on a confidence level of 95%, a precision of 5%, and sensitivity/specificity data from previous studies. The calculated sample size for the study was 320 participants.

Data Collection: Demographic and clinical data, including age, gender, height, weight, Body Mass Index (BMI), duration of diabetes, and other relevant medical history, were collected from the hospital information system.

Laboratory investigations included:

- Biochemical Parameters: Fasting plasma glucose, HbA1c, serum urea, serum creatinine, calculated eGFR, serum cholesterol, serum triglycerides, serum LDL-c, serum HDL-c, UACR
- Urinary tubular markers: NGAL, Megalin, NAG, Cystatin-c

Analytical methods:

For routine biochemical parameters:

Parameter	**Sample used**	**Method**	**Analyzer**
Glucose	Plasma (Fluoride tube)	Hexokinase	Cobas 6000
HbA1c	Plasma (EDTA tube)	Immunoturbidimetry	Cobas Integra
Urea	Serum (Plain tube)	Kinetic test with urease and glutamate dehydrogenase	Cobas 6000
Creatinine	Serum (Plain tube)	Enzymatic, colorimetric	Cobas 6000
Cholesterol	Serum (Plain tube)	Enzymatic, colorimetric	Cobas 6000
Triglyceride	Serum (Plain tube)	Enzymatic, colorimetric	Cobas 6000
HDL-c	Serum (Plain tube)	Enzymatic, colorimetric	Cobas 6000
LDL-c	Serum (Plain tube)	Enzymatic, colorimetric	Cobas 6000
Urine albumin	Urine	Immunoturbidimetry	Cobas 6000
Urine creatinine	Urine	Enzymatic, colorimetric	Cobas 6000

For urine tubular biomarkers: The urinary biomarkers were measured using commercially available ELISA kits. The levels of Neutrophil Gelatinase-Associated Lipocalin (NGAL), Megalin, and N-Acetyl-β-D-Glucosaminidase (NAG) were estimated using ELISA kits from Abbkine (Abbkine Scientific Co., Ltd., China), while Cystatin-C was measured using an ELISA kit from Elabscience (Elabscience Biotechnology Inc., China). The assays were performed according to the manufacturer's protocols.

e-GFR estimation

The CKD-EPI 2021 equation is: eGFR = 142 x min (standardized Scr/K,1) $^{\alpha}$ * max (standardized Scr/K,1) $^{-1.200}$ * 0.9938 $^{\text{age in years}}$ * 1.012 [if female]

where: Scr = serum creatinine in mg/dL; K = 0.7 (females) or 0.9 (males); α = -0.241 (females) or -0.302 (males); min(standardized Scr/K,1) = the minimum of Scr/K or 1; max(standardized Scr/K,1) = the maximum of Scr/K or 1

MDRD Study Equation

eGFR = 175 x $(S_{Cr})^{-1.154}$ x $(age)^{-0.203}$ x 0.742 [if female] x 1.212 [if Black]

eGFR (estimated glomerular filtration rate) = mL/min/1.73 m^2

S_{cr} (standardized serum creatinine) = mg/dL

Statistical Analysis:

Prior to analysis, normality of data distribution was assessed using the Shapiro-Wilk test. Given the non-normal distribution of biomarkers (all p-values < 0.05), non-parametric statistical methods were selected to ensure statistical validity.

Descriptive Statistics

- Summarized baseline characteristics of the study population
- Calculated mean, standard deviation, minimum, and maximum values for each biomarker across different albuminuria groups

Group Comparisons

- Utilized Kruskal-Wallis test to assess significant differences in biomarker levels across normoalbuminuria, microalbuminuria, and macroalbuminuria groups
- Identified statistically significant variations in biomarker concentrations

Diagnostic Performance Evaluation

- Conducted Receiver Operating Characteristic (ROC) curve analysis
- Calculated Area Under the Curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy for each biomarker
- Determined optimal diagnostic cut-off values for distinguishing between normoalbuminuria and micro/macroalbuminuria

Correlation Analysis

- Employed Spearman's correlation coefficient to examine relationships between: a) Urinary biomarkers and clinical parameters (HbA1c, diabetes duration, urea, creatinine, lipid profile) b) Estimated glomerular filtration rate (eGFR) using MDRD and CKD-EPI 2021 equations and urinary biomarkers

Predictive Modelling

- Applied multiple logistic regression to: a) Assess independent predictive power of biomarkers and clinical parameters b) Classify albuminuria status c) Calculate adjusted odds ratios (OR) d) Determine statistical significance of predictors

All statistical analyses were performed using IBM SPSS Statistics (Version 26.0). Statistical significance was set at $p < 0.05$, with a 95% confidence interval.

RESULTS & DISCUSSION

A total of 320 participants were included in the study. They were divided into three groups based on their urinary albumin creatinine ratio (UACR) as follows:

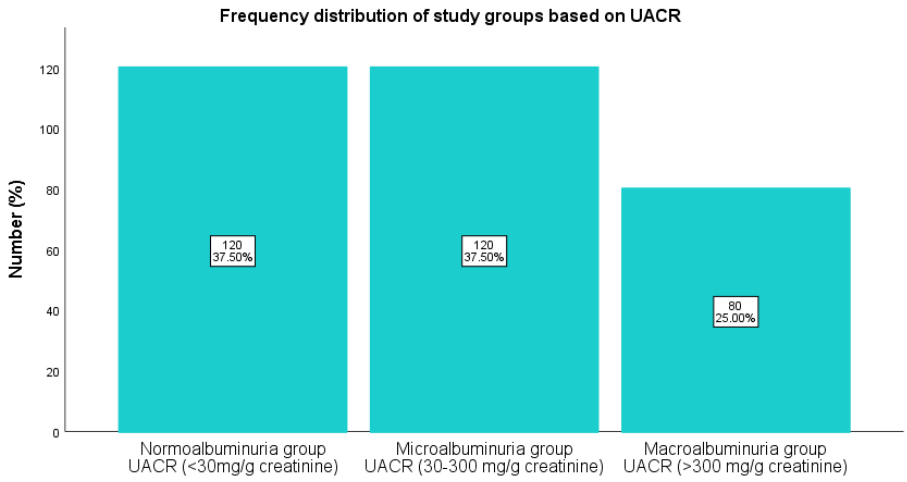
Group-1: Normoalbuminuria group (UACR <30 mg/g creatinine)

Group-2: Microalbuminuria group (UACR 30-300 mg/g creatinine)

Group-3: Macroalbuminuria group (UACR >300 mg/g creatinine).

The participants were strategically distributed across three albuminuria categories to enable comprehensive evaluation of biomarker performance across different stages of DKD. (Figure 1)

Figure 1: Frequency distribution of participants into study groups



Shapiro-Wilk normality testing for NAG, Megalin, NGAL, and Cystatin-c revealed p-values < 0.05; this indicated that none of the biomarkers followed normal distribution. Megalin ($p = 3.04E^{-13}$), NGAL ($p = 8.22E^{-07}$) and Cystatin-c ($p = 2.17E^{-09}$) showed strong deviations from normality which confirmed skewed distributions. Hence non-parametric tests such as Spearman's

correlation, Mann-Whitney U tests and Kruskal-Wallis were chosen to ensure statistical validity in analysing biomarker differences across groups.

The Kruskal-Wallis test was performed to assess whether significant differences existed in biomarker levels between groups. The results provide an overview of the distribution and variability of these biomarkers in Table-1.

Table-1: Comparison of urinary biomarkers across study groups

Biomarker	Group	Min	Mean ± SD	Max	p-value
NAG	1	15.18	58.10 ± 22.62	122.77	< 0.0001*
	2	27.12	79.20 ± 20.41	109.46	
	3	15.18	69.74 ± 23.93	120.08	
Megalin	1	5.37	17.20 ± 8.12	43.53	0.0001*
	2	5.01	12.82 ± 4.60	26.91	
	3	5.18	14.46 ± 6.95	41.54	
NGAL	1	0.78	1.79 ± 0.59	3.23	< 0.0001*
	2	1.04	2.58 ± 0.63	3.53	
	3	0.79	2.22 ± 0.70	3.52	
Cystatin-c	1	0.48	14.78 ± 7.17	26.01	0.0949
	2	4.91	16.60 ± 2.72	23.43	
	3	1.01	15.46 ± 5.14	25.95	

*p-value <0.05 – statistical significance

The significant differences observed in NAG, Megalin, and NGAL levels confirm their potential utility in detecting early kidney damage before overt albuminuria appears, aligning with the

hypothesis that tubular biomarkers are more effective than UACR in early detection of diabetic nephropathy. However, Cystatin-c did not show significant group-wise variation; this indicated its role may be limited or influenced by other factors.

Diagnostic performance of urinary biomarkers

Urinary biomarkers (NAG, Megalin, NGAL, and Cystatin-c) were evaluated for their ability to distinguish normoalbuminuria (UACR < 30 mg/g) from microalbuminuria/macroalbuminuria (UACR ≥ 30 mg/g) using Receiver Operating Characteristic (ROC) curve analysis. AUC (Area Under the Curve) values were calculated for each biomarker to determine its diagnostic accuracy in identifying albuminuria progression (Table-2 and Figure 2).

Table-2: Diagnostic performance of urinary biomarkers

Biomarker	AUC	Sensitivity	Specificity	PPV	NPV	Accuracy
NAG	0.719	0.815	0.600	0.773	0.661	0.734
Megalin	0.363	0.000	1.000	0.000	0.375	0.375
NGAL	0.764	0.625	0.817	0.850	0.566	0.697
Cystatin-c	0.552	0.845	0.458	0.722	0.640	0.700

The AUC analysis using UACR groups confirmed that NAG and NGAL exhibit strong diagnostic potential, with NGAL showing higher specificity and positive predictive value. Cystatin-c demonstrated moderate predictive ability, while Megalin performed poorly, indicating limited relevance as a diagnostic biomarker for albuminuria.

N-Acetyl Glucosaminidase (NAG)

- AUC: 0.719 indicated strong diagnostic performance, confirming its ability to distinguish between normoalbuminuria and micro/macroalbuminuria.
- Sensitivity: 0.815, suggesting high ability to correctly detect albuminuric patients.
- Specificity: 0.600, indicating moderate ability to correctly classify normoalbuminuric individuals.
- PPV (Positive Predictive Value): 0.773, meaning high likelihood that a positive test corresponds to true albuminuria.
- NPV (Negative Predictive Value): 0.661, showing moderate confidence in negative test results ruling out albuminuria.
- Accuracy: 0.734, supporting its overall reliability as a predictive biomarker.

Megalin

- AUC: 0.363, indicating poor classification performance, suggesting that Megalin may not be effective as a standalone diagnostic marker for albuminuria.
- Sensitivity: 0.000, meaning no cases of albuminuria were correctly identified.
- Specificity: 1.000, implying perfect ability to identify normoalbuminuric individuals but with no ability to detect disease.
- PPV: 0.000, reinforcing no predictive power for positive test results.
- NPV: 0.375, suggesting low confidence in negative test results.
- Accuracy: 0.375, indicating very limited clinical utility.

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

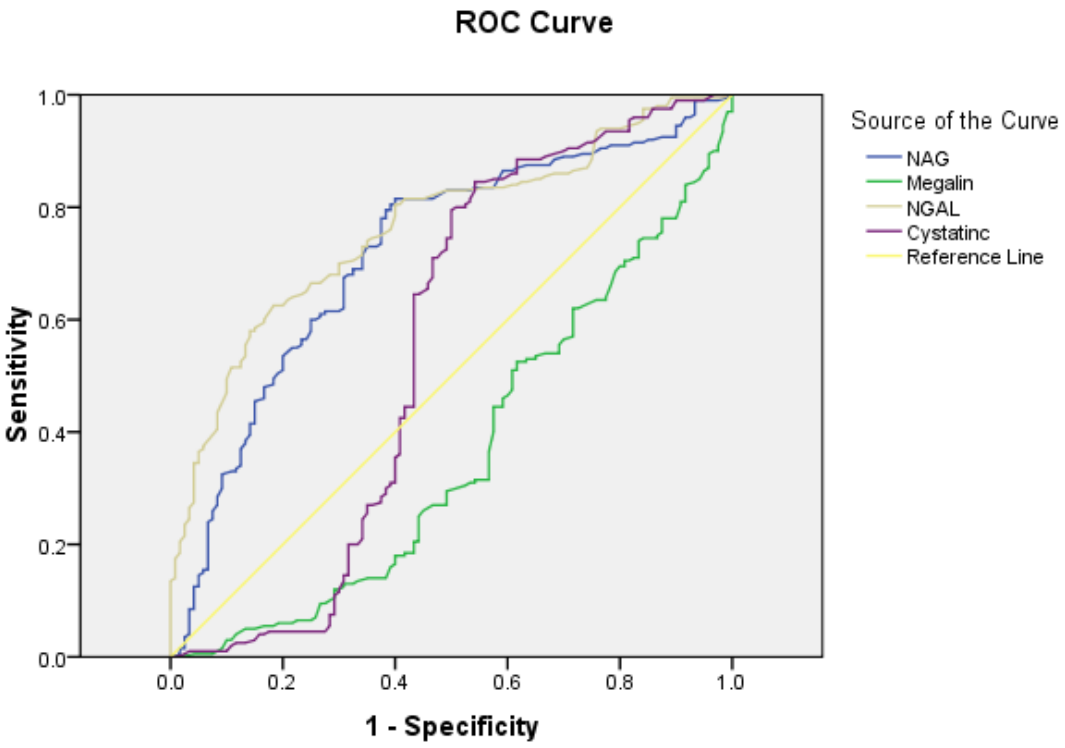
- AUC: 0.764, suggesting strong diagnostic capability and confirming NGAL's potential utility as an early marker of albuminuria progression.
- Sensitivity: 0.625, indicating moderate ability to detect albuminuria cases.
- Specificity: 0.817, demonstrating high ability to correctly classify normoalbuminuric individuals.
- PPV: 0.850, meaning high likelihood that a positive test corresponds to true albuminuria.
- NPV: 0.566, suggesting moderate reliability in ruling out disease.
- Accuracy: 0.697, supporting its role as a potential screening tool.

Cystatin-c

- AUC: 0.552, indicating borderline diagnostic performance and suggesting limited utility as a predictive marker.
- Sensitivity: 0.845, meaning high ability to detect albuminuria cases.
- Specificity: 0.458, indicating low ability to correctly classify normoalbuminuric individuals.
- PPV: 0.722, implying moderate confidence in positive test results.
- NPV: 0.640, suggesting reasonable reliability in negative test results.
- Accuracy: 0.700, showing moderate predictive power.

These findings support the hypothesis that tubular biomarkers like NAG and NGAL could enhance early detection of diabetic nephropathy; they could supplement or improve upon UACR alone.

Figure 2: Receiver Operating Characteristic (ROC) Curves for Urinary Biomarkers in Detecting Diabetic Kidney Disease



Correlational analysis examined relationships between urinary biomarkers (NAG, Megalin, NGAL, Cystatin-c) and other study parameters (HbA1c, Duration of Diabetes, Urea, Creatinine, LDLc, Triglycerides). Correlation coefficients and statistical significance (p-values) were computed to evaluate associations in Table-3.

Table-3: Correlation analysis between urinary biomarkers and study parameters

Biomarker	Clinical Parameter	Correlation Coefficient	p-value
NAG	HbA1c	0.0719	0.1993
	Duration of DM	-0.0407	0.4683
	Urea	-0.0472	0.3999
	Creatinine	0.1266	0.0235*
	LDL-c	0.1439	0.0100*
	HDL-c	0.0295	0.5994
	Cholesterol	0.0345	0.5391
	Triglyceride	0.0688	0.2196
Megalin	HbA1c	-0.0007	0.9900
	Duration of DM	0.1193	0.0328*
	Urea	-0.0532	0.3424
	Creatinine	-0.0813	0.1467
	LDL-c	-0.0295	0.5996
	HDL-c	0.0224	0.6889
	Cholesterol	-0.0955	0.0881
	Triglyceride	-0.0793	0.1570
NGAL	HbA1c	0.2135	0.0001*
	Duration of DM	0.0695	0.2153
	Urea	0.0472	0.4003
	Creatinine	0.0557	0.3203
	LDL-c	0.1173	0.0360*
	HDL-c	0.0775	0.1665
	Cholesterol	-0.0022	0.9682
	Triglyceride	0.1411	0.0115*
Cystatin-c	HbA1c	0.0923	0.0994
	Duration of DM	0.1050	0.0607
	Urea	0.0588	0.2942
	Creatinine	0.0105	0.8514
	LDL-c	-0.0138	0.8056
	HDL-c	-0.0277	0.6216
	Cholesterol	-0.1412	0.0115*
	Triglyceride	0.0684	0.2222

*p-value <0.05 – statistical significance

Correlation between eGFR estimation methods and urinary biomarkers

eGFR values estimated using MDRD and CKD-EPI 2021 equations correlated with urinary biomarkers NAG, Megalin, NGAL, and Cystatin-c; Spearman's correlation coefficients and p-values calculated to assess strength and significance of associations (Table-4).

Table-4: Correlation analysis between urinary biomarkers and eGFR

eGFR Method	Biomarker	Spearman Correlation Coefficient	p-value
MDRD	NAG	-0.1289	0.0211*
	Megalin	0.0699	0.2124
	NGAL	-0.0732	0.1917
	Cystatin-c	-0.0411	0.4636
CKD-EPI 2021	NAG	-0.1122	0.0449*
	Megalin	0.0469	0.4028
	NGAL	-0.0722	0.1977
	Cystatin-c	-0.0541	0.3343

*p-value <0.05 – statistical significance

MDRD showed weak negative correlation with NAG (ρ = -0.1289, p = 0.0211); weak positive correlation with Megalin (ρ = 0.0699, p = 0.2124); weak negative correlations with NGAL (ρ = -0.0732, p = 0.1917) and Cystatin-c (ρ = -0.0411, p = 0.4636). CKD-EPI 2021 showed weak negative correlation with NAG (ρ = -0.1122, p = 0.0449); correlations with other biomarkers not statistically significant (p > 0.05).

Multiple logistic regression analysis aimed to determine whether a combination of urinary biomarkers (NAG, Megalin, NGAL, Cystatin-c) and clinical parameters (HbA1c, Duration of Diabetes, Urea, Creatinine, LDLc, Triglycerides) can effectively classify albuminuria status (normoalbuminuria vs micro/macroalbuminuria). A multiple logistic regression model was applied and adjusted for confounding variables - to assess the independent predictive power of each factor. Odds ratio (OR) and p-values were computed to evaluate the strength and significance of associations. The accuracy, sensitivity, specificity and AUC (Area Under the Curve) were assessed to determine the model's diagnostic capability.

NGAL (OR = 1.892, p = 0.005) and NAG (OR = 1.043, p = 0.018) were strong independent predictors of albuminuria; supported hypothesis tubular biomarkers provide diagnostic value beyond UACR. HbA1c (OR = 1.354, p = 0.029) and Creatinine (OR = 1.268, p = 0.036) were significant predictors; reinforced association between glycemic control, renal function, and albuminuria progression. Urea (OR = 1.015, p = 0.011) showed significant contribution; confirmed role in renal function assessment. Megalin (p = 0.602), Cystatin-c (p = 0.408), LDL-c (p = 0.591), and Triglycerides (p = 0.482) did not show significant predictive value; suggested limited role in albuminuria classification.

This cross-sectional study provides important insights into the utility of urinary tubular biomarkers for early detection of DKD in Type-2 diabetes mellitus patients. The analysis of 320 participants across different albuminuria stages revealed several significant findings that contribute to our understanding of tubular injury in DKD progression.

The study demonstrated that urinary tubular biomarkers, particularly NAG and NGAL, show significant alterations even in early stages of kidney dysfunction. The ROC analysis revealed that NGAL exhibited the strongest diagnostic performance (AUC 0.76) among the novel biomarkers,

followed by NAG (AUC 0.72). These findings align with recent studies by Zhang et al. (2024) and support the hypothesis that tubular injury may precede significant glomerular dysfunction in diabetic nephropathy.

Interestingly, the correlation analysis revealed significant associations between certain biomarkers and clinical parameters. NGAL showed a positive correlation with HbA1c ($p=0.008$), suggesting a potential link between glycemic control and tubular injury. Cystatin-c demonstrated correlations with both age ($p=0.019$) and diabetes duration ($p=0.022$), indicating its potential value in monitoring disease progression over time.

The observation that these biomarkers show alterations in the microalbuminuria group, and sometimes even in the normoalbuminuria group, supports their potential utility in early detection of kidney injury. This is particularly relevant given that traditional markers like serum creatinine and eGFR often remain normal until significant kidney damage has occurred.

SUMMARY AND CONCLUSION

This comprehensive cross-sectional study investigated the potential of urinary tubular biomarkers in early detection of DKD among type-2 diabetes mellitus patients. By analysing 320 participants across different albuminuria stages, the research highlighted critical insights into kidney injury detection beyond traditional screening methods.

The key findings reveal that urinary biomarkers, particularly NGAL and NAG, demonstrate remarkable potential for early DKD detection. NGAL exhibited the most promising diagnostic performance, with an Area Under the Curve (AUC) of 0.764 and high specificity of 0.817. Notably, these biomarkers showed significant alterations even in early stages of kidney dysfunction, often preceding changes detectable by conventional markers like UACR.

Multiple logistic regression confirmed NGAL and NAG as strong independent predictors of albuminuria, with significant correlations to clinical parameters such as HbA1c. This suggests a complex interplay between glycemic control and kidney injury that extends beyond traditional understanding.

While promising, these findings necessitate further longitudinal research to validate the biomarkers' clinical utility and establish definitive diagnostic cut-off values. The multi-marker approach proposed here represents a significant step towards more sensitive and proactive kidney disease screening in diabetic patients.

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PUBLICATION COPY FIRST PAGE – ARTICLE:1

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Research Article

Does Measuring Urinary Tubular Biomarkers Have Diagnostic Potential for Detecting Early Diabetic Nephropathy in Type-2 Diabetes Mellitus Patients?

Sujatha Rajaragupathy[1*], Sumathi S[2], Vijaya Duraisamy[3], Saju Denishya Prabhakaran[4], Susipriya Palanisamy[5], Aathira Sunilkumar[6]

[1*] Associate Professor, Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore, IND & Part time PhD student (external), Sri Balaji Vidyapeeth Deemed-to-be-University, Pondicherry, IND
[2] Professor and Head, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth Deemed-to-be-University, Pondicherry, IND
[3] Professor, Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore, IND
[4] Undergraduate MBBS Student, Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore, IND
[5] Center for Molecular Medicine and Therapeutics, PSG Institute of Medical Sciences and Research, Coimbatore, IND
[6] Undergraduate MBBS Student, Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore, IND

***Corresponding author:** Sujatha R
*Email; suja1357@gmail.com

Abstract

Background: Higher prevalence and mortality in type-2 diabetes mellitus (T2DM) patients due to diabetic nephropathy (DN) highlights the need for determining early-stage DN biomarkers for better diagnosis and prognosis.

Objectives: To evaluate the diagnostic potential of urinary markers of tubular injury- megalin, neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-beta-D-glucosaminidase (NAG), and cystatin C to determine early DN against Urine Albumin Creatinine Ratio.

Methods: This cross-sectional study conducted between during 2022-2023, included 83, T2DM patients attending a tertiary care hospital in south India. Their baseline clinical parameters were recorded, and urine samples were obtained to determine the different biomarkers present using enzyme-linked immune-sorbent assay (ELISA) and the receiver operating curve (ROC) was constructed to determine the cut-off values for urinary markers. A p-value<0.05 was considered statistically significant.

Results: Of 83 patients with a mean T2DM duration of 7.1±3.5 years were enrolled, 55.4% were normal, 38.6% had microalbuminuria, and 6% had macroalbuminuria. Megalin was significantly different among the three groups, while other urinary biomarkers failed to show any significant difference. The Area under curve value for Megalin (AUC=0.640, 95% confidence interval, CI=0.521 to 0.758) was found to be statistically significant in differentiating macroalbuminuric patients from the other two groups (p=0.03). The specificity and sensitivity values were variable and moderate including Megalin.

Conclusion: While megalin demonstrated notable differences, other biomarkers did not vary significantly among the groups of patients with different UAE ratios. Moderate sensitivities and specificities suggest that these biomarkers cannot be used as a standalone diagnostic method for DN, and more research should be conducted to validate these results.

Categories: Endocrinology/Diabetes/Metabolism, Internal Medicine, Nephrology
Keywords: type 2 diabetes mellitus, megalin, diabetic nephropathy, biomarkers, albuminuria

***Author for correspondence*: *Email:** *suja1357@gmail.com*

PUBLICATION COPY FIRST PAGE – ARTICLE:2

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Comparative utility of urinary Megalin and Cystatin-c in identifying early renal damage in Type-2 Diabetes mellitus – A cross sectional study

Sujatha Rajaragupathy[1], Sumathi S[2], Vijaya Duraisamy[3], Susipriya Palanisamy[4], Saju Denishya[5], Aathira Sunilkumar[6], Dharshika Saravanan[7]

[1]*Biochemistry, PSG Institute of Medical Sciences and Research, Part time PhD student at Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry*

[2]*Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry*

[3,5,6,7]*Biochemistry, PSG Institute of Medical Sciences and Research*

[4]*Centre for Molecular Medicine & Therapeutics, PSG Institute of Medical Sciences and Research*

Corresponding Author- *Sujatha Rajaragupathy

PSG Institute of Medical Sciences and Research, Coimbatore, Tamilnadu, India

Abstract-Kidney disease is a major complication of type-2 diabetes mellitus (T2DM) and a leading cause of end-stage renal disease. Early detection is crucial to prevent the progression of kidney damage. However, current markers such as microalbuminuria have low specificity, necessitating the exploration of novel biomarkers. Urinary megalin and cystatin-C have been proposed as potential early indicators of renal damage. The aim of the study is to evaluate urinary megalin and cystatin-C as biomarkers for early detection of renal damage in T2DM patients with different levels of albuminuria.

This diagnostic evaluation study included 120 T2DM patients from a tertiary care hospital in South India, recruited between January and June 2023. Patients were grouped based on their urinary albumin-creatinine ratio (ACR): normoalbuminuria (ACR <30 mg/g) and microalbuminuria (ACR 30-299 mg/g). Urinary megalin and cystatin-c levels were measured using ELISA kits, and their associations with glycemic control, lipid profile, and other parameters were analyzed using Pearson's correlation.

The microalbuminuria group showed significantly lower urinary megalin (15.47 ± 4.99 pg/mL vs. 17.40 ± 5.11 pg/mL, p = 0.04) and cystatin-c levels (14.20 ± 7.87 ng/mL vs. 17.21 ± 7.60 ng/mL, p = 0.035) than the normoalbuminuria group.

Urinary megalin and cystatin-c are promising early biomarkers for detecting renal damage in T2DM patients, potentially improving early diagnosis and outcomes.

Keywords: Diabetic Nephropathy; Type-2 Diabetes Mellitus; Biomarkers; Urinary Megalin; Urinary Cystatin-C; Microalbuminuria; Early Detection; Renal Damage.

Contribution details

concepts, design, definition of intellectual content, literature search, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review.

INTRODUCTION

Kidney disease is a prevalent complication of type-2 diabetes mellitus (T2DM) and a leading cause of end-stage renal disease (ESRD) worldwide (Eckardt et al., 2013). Early detection of renal damage in T2DM patients is crucial to prevent progression to chronic kidney disease (CKD) and its associated complications (Thomas et al., 2015). Currently, microalbuminuria, measured by the urine albumin-creatinine ratio (ACR), is the most widely used biomarker for detecting early diabetic kidney damage. However, its limitations, such as low specificity and the influence of non-renal factors, necessitate the identification of novel biomarkers for more accurate early detection of renal dysfunction (Perkins et al., 2007).

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Research Article

Association of Albumin-to-Creatinine Ratio with Dyslipidemia and Glycemic index in Type 2 Diabetes Mellitus – A cross-sectional study

Sujatha Rajaragupathy[1*], Sumathi S[2], Vijaya Duraisamy[3], Susipriya Palanisamy[4], Saju Denishya[5], Aathira Sunilkumar[6], Nayanthra Karthik[7], Ashvanthidhaa Jayaraman[8]

[1*]PSG Institute of Medical Sciences and Research Part time PhD student at Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry, Biochemistry
[2]Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry Biochemistry
[3]PSG Institute of Medical Sciences and Biochemistry Research
[4]PSG Institute of Medical Sciences and Research, Centre for Molecular Medicine & Therapeutics
[5]PSG Institute of Medical Sciences and Research. Biochemistry
[6]PSG Institute of Medical Sciences and Research Biochemistry
[7]PSG Institute of Medical Sciences and Research Biochemistry
[8]PSG Institute of Medical Sciences and Research Biochemistry

***Corresponding Author:** Sujatha Rajaragupathy
*PSG Institute of Medical Sciences and Research Coimbatore, Tamilnadu, India Telephone: (91)-9487539777. Email ID: sujatha.biochemistry@gmail.com, Department of Biochemistry, PSG Institute of Medical Sciences and Research

Summary:
Type 2 diabetes mellitus (T2DM) patients have a high prevalence of dyslipidemia and renal impairment, increasing cardiovascular and renal risk. Albumin-creatinine ratio (ACR) is a marker for early renal impairment, while lipid disorders with elevated LDL-C and triglycerides are common in T2DM. This study aimed to explore the relationship between ACR and lipid profiles. In the retrospective analysis of 300 T2DM patients, ACR showed a weak but significant correlation with total cholesterol ($r = 0.117$, $p = 0.043$) and stronger correlations with HbA1c ($r = 0.183$, $p = 0.001$) and postprandial glucose ($r = 0.154$, $p = 0.007$). Subgroup analysis identified the 41–60 age group as having the highest mean ACR (123.50 mg/g) and triglycerides (147.50 mg/dL). High ACR levels (≥30 mg/g) were linked to low total cholesterol and LDL-C, likely due to lipid-lowering therapies. These findings emphasize integrated monitoring of renal, lipid, and glycemic parameters to mitigate risks of nephropathy and cardiovascular disease.

Keywords: albumin-to-creatinine ratio, lipid profile, renal dysfunction, cardiovascular risk, lipid-lowering therapies.

***Author of Correspondence: Email:** sujatha.biochemistry@gmail.com*

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Urinary Megalin as a Potential Biomarker for Early Detection of Kidney Damage in Diabetic Nephropathy

at the Conference on 5th & 6th September 2024, organised by the Department of Community Medicine & Medical Education Unit, Government Medical College, Omandurar Government Estate, Chennai.

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